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Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine

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An experimental serogroup B meningococcal vaccine was prepared from two genetically engineered strains; each expressing three different class 1 outer membrane proteins (OMPs) (PorA). The two strains expressed the subtypes P1.7,16;P1.5,2;P1.19,15 and P1.5^c,10;P1.12,13;P1.7^h,4, respectively. Outer membrane vesicles (OMV) were prepared from these strains by deoxycholate extraction, mixed with aluminiumphosphate as adjuvant and formulated to final vaccines. The class 1 OMPs represent ca *90% of the protein in the vaccine. The vaccine was found safe for human use and induced a bactericidal immune response in mice against five of the six wild type strains, which served as donors for the various porA genes. Copyright © 1996 Elsevier Science Ltd.*

Neisseria meningitidis serogroup B strains are responsible for a large number of clinical cases of bacterial meningitis in the Netherlands and worldwide[1,2]. General immunization of the population appears to be the only means to reduce the mortality and morbidity due to meningococcal disease, beyond antibiotic therapy.

The B capsular polysaccharide, being the first choice as a vaccine component, was found to be nonimmunogenic[3]. After chemical modification and conjugation to a carrier protein the polysaccharide was found to be immunogenic in animals[4]. Results with volunteers are not yet described. The issue of safety, however, needs to be addressed. The B polysaccharide is structurally identical to saccharide containing components such as N-CAM in man and mammals. Currently more experience has been obtained with vaccines which are based on meningococcal outer membrane proteins (OMPs). A number of large scale efficacy trials have been carried out with meningococcal OMPs either purified[5] or in an outer membrane vesicle (OMV) formulation[6]. In each instance, the OMPs were derived from wild type strains and the vaccines were designed to combat occurring epidemics. These vaccines, contain multiple OMPs derived from one meningococcus. These OMPs are mainly the class 1, 2 or 3, 4 and 5 proteins. Such first generation OMP vaccines demonstrated efficacies around 50–80% except in infants were their efficacy is negligible[7].

In vitro bactericidal antibody assays were found to be partly strain specific, revealing a particular importance of the class 1 OMP[8]. These findings are in line with immunogenicity and protection data in mice and in rats[9]. The extent of heterogeneity of the class 1 OMP is limited[10] which motivated us to develop multivalent class 1 OMP containing meningococcal OMV vaccines. We constructed a number of vaccine strains which are able to express three class 1 OMPs[11,12]. The expression of Class 3 OMP was eliminated because this protein contributes little to the induction of bactericidal antibodies in mice and man[8,9]. The B-polysaccharide and lacto-*N*-tetraose structures are deleted since those are shared with host structures and immune responses against them are poor and may even be deleterious. The expression of Opc is left untouched, since this protein may induce cross reactive bactericidal antibodies[8], although the expression is poor by many case isolates[13]. The heterogeneity and variability of Opa proteins appears very high[14] so we did not attempt to realise a high expression, although some Opa is left in our vaccine. The six PorA proteins chosen, which constitute about 90% of the vaccine protein, were chosen on the basis of epidemiological data from the Netherlands[1] but also cover the majority of a global strain collection[10]. A major issue to be resolved remains the occurrence

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of microheterogeneity, i.e. point mutations/deletions, within PorA and the cross reactivity of human polyclonal antibodies to such heterogeneity[15]. This issue will be addressed by clinical studies with the vaccine described. In addition to PorA, other major OMP exist that bind/induce bactericidal antibodies such as Tbp-2 and FrpB. These proteins reveal strong heterogeneity[16,17] which currently is not understood in enough detail. Meningococcal vaccine development in essence is still a matter of debate. We have made a first choice for a set of proteins on the basis of combined epidemiological, genetical, structural and immunological data.

In this paper we describe the production and control of an experimental hexavalent class 1 OMP containing (P1.7,16;P1.5,2;P1.19,15; P1.5^c,10;P1.12,13;P1.7^h,4) OMV vaccine using two vaccine strains. Bacteria are treated with deoxycholate (DOC) to extract OMVs. The OMVs were isolated and mixed with aluminumphosphate as adjuvant. The vaccines made thereof were found to be safe and immunogenic and suitable for human use. A safety study with adult volunteers has been carried out and will be presented in a follow up paper[18].

MATERIALS AND METHODS

Bacterial strains

Two *N. meningitidis* strains PL16215 (CPS$^-$, P1.7,16,5,2,19,15) and PL10124 (CPS$^-$, P1.5^c,10,12,13,7^h,4)[12] were used for the production of the OMV-vaccine.

Vesicle production

Hexavalent meningococci-B vaccine was produced using two different *N. meningitidis* trivalent strains PL16215 and PL10124, each producing three different class 1 proteins.

The cultivation medium was meningococci medium according to Frantz, with main components: L-glutamic acid, L-cysteine, glucose and yeast extract[19]. Cells were cultivated at 36°C for 18 h, in a 135 l volume.

The cell harvest was concentrated by centrifugation by continuous flow centrifugation (Westfalia separator) and the cells were resuspended in NaCl buffer. The cell suspension was homogenized for 30 min and the total wet weight of the suspension was determined The cell suspension was centrifuged for 60 min at 2900 *g*. The cell pellet was resuspended in 0.1 M Tris–10 mM EDTA-buffer, 7.5 times wet weight. Extraction of the vesicles was performed by the addition of 1/20th volume of 0.1 M Tris, 10 mM EDTA, DOC (100 g l^{-1}) buffer. Vesicles were separated from cell debris at 20000 *g* at 4°C for 1 h (Centrikon T-1170). The supernatant containing the vesicles was concentrated by ultracentrifugation at 125000 *g* at 4°C for 2 h. The OMV pellet was resuspended in 0.1 M Tris, 10 mM EDTA, DOC (5 g l^{-1}) buffer and the suspension was centrifuged again at 125000 *g* at 4°C for 2 h. The concentrated OMV were resuspended in 3% sucrose solution[20]. The two trivalent bulk products were mixed in equimolar amounts based on class 1 OMP protein content with $AlPO_4$ as adjuvant. Throughout the process thiomersal (100 mg l^{-1}) was added as preservative.

Electron microscopy and immunogold labelling

OMVs were ultrasonically treated to disperse the vesicles and were attached to Formvar/carbon-coated nickel grids. Grids were washed with a 0.01 M PBS supplement with 0.5% BSA and 0.1% gelatine (PBG) and the vesicles on the grids were fixed briefly with 1% glutaraldehyde in PBS and negatively stained with 1% potassium phosphotungstate pH 6.0. The grids were examined in a Philips EM400T electron microscope at 80 kV.

For immunogold labelling grids were washed with 5% BSA+1% normal goat serum in 0.01 M PBS followed with PBG to avoid aspecific background labelling. The grids were incubated at 4°C for 16–18 h with anti-OMP monoclonal antibodies diluted in PBG. After washing with PBG the vesicles were labelled with goat-anti-mouse IgG conjugated to 6 nm colloidal gold (GAMG6) (Aurion, Wageningen, The Netherlands), diluted 1:20 in PBG, for 4 h at 20°C. Control incubations were carried out with irrelevant monoclonal antibodies also diluted in PBG.

The grids were washed again with PBG and the vesicles on the grids were fixed briefly with 1% glutaraldehyde in PBS, washed in PBG, and negatively stained as described.

Protein determination

Protein concentration was measured according to Peterson[21]. Briefly vesicles were solubilized in the presence of 0.15% (w/v) DOC for 10 min. Proteins were precipitated by the addition of 72% (w/v) trichloroacetic acid (TCA) and centrifuged at 3000*g* for 15 min at 4°C. The pellet was dissolved in distilled water. The protein content was determined using copper–tartrate–carbonate and Folin Ciocalteu's phenol reagent. The absorption at 750 nm was determined after 30 min incubation at RT and compared to a bovine serum albumin standard (Pierce, USA).

Monoclonal antibodies

Monoclonal antibodies (MoAbs) against individual subtype specific epitopes[10], different OMPs and mutant lipopolysaccharide (LPS) were used in ELISA, immunoblotting and immunogold EM. A list of the MoAbs used and the antigens which they recognise is given in *Table 1*.

SDS–PAGE, quantitation of protein bands and immunoblotting

Vaccine samples were analysed on 10% polyacrylamide gels in the presence of 2% (w/v) SDS[22]. After electrophoresis proteins were stained by silver staining[23] or by 0.1% (w/v) Coomassie Brilliant Blue staining. The relative amount of class 1 OMP in the vaccine was determined by staining the gel using Coomassie Brilliant Blue. The intensity of Coomassie Brilliant Blue stained protein bands was determined by gelscanning and the relative amount of class 1 OMP is calculated using Imagemaster (Pharmacia, Sweden) software and given as percentage of the total protein content.

Unstained proteins were transferred to nitrocellulose sheets by electroblotting. Proteins were visualized using

Table 1 The MoAbs and the meningococcal antigens that they recognize

MoAb	Protein specificity
MN14C11.6	P1.7
MN5C11G	P1.16
MN22C2.55	P1.5
MN16C13F4	P1.2
MN3C5C	P1.15
MN20F4.17	P1.10
MN20B9.34	P1.4
MN20A7.10	P1.12
MN24H10.75	P1.13
MN15A14H6	Class 3 OMP
MN3B9F	Class 4 OMP
15-1P5.5	Class 5 OMP, specificity 5.5
S3141	Opc
4A8B2	L3 LPS
MN31D8.51	galE LPS

These MoAbs have been used for ELISA, immunoblotting analysis and immunogold EM

type specific MoAb (*Table 1*) or sera derived from mice and Protein-A-HRP conjugate (Amersham, UK).

Identity test by ELISA

The identity of the vaccine was determined by identification of characteristic epitopes using ELISA. Starting at a concentration of 0.3 μg class 1 OMP per well ^{2}log dilution's of the vesicles were coated in PBS 0.01 M pH 7.2 on PVC 96-well plates (Greiner) overnight at room temperature. The coated plates were incubated at 37°C for 1 h with subtype-specific monoclonal antibodies in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA (*Table 1*). The binding of MoAbs was demonstrated using biotinylated goat-anti-mouse-IgG (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 1 h and streptavidin horseradish peroxidase (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 30 min. Binding of the conjugate was visualized using TMB/ethanol substrate. Binding of specific monoclonal antibody should be demonstrated, whereas binding of non-specific antibody (directed against subtypes of the other strain) should be absent.

LPS determination

The LPS content was determined by SDS–PAGE[24]. Vesicles were dissolved in a buffer containing 2% (v/v) β-mercapto-ethanol and 2% (w/v) SDS and boiled for 5 min followed by proteinase-K (2.5 mg ml^{-1}, Boehringer) treatment for 1 h at 60°C. The treated samples were separated by SDS–PAGE with a 14% polyacrylamide gel. LPS was oxidized with 0.4% (w/v) periodic acid and visualized by silver staining. Unknown LPS samples were compared with an LPS standard in the same gel. LPS concentration was also determined by gaschromatography[25].

Endotoxin determination

The biological activity of the endotoxin was determined in the limulus amebocyte lysate (LAL) assay. The lipid-A part of the endotoxin molecule can activate the gelation of the limulus lysate. The OMV vaccine was incubated in a fivefold dilution with a fixed concentration of limulus lysate (Heamachem Inc., St. Louis, USA) at 37°C for 45 min. The endotoxin activity was visualized using Bromthymol Blue. Unknown activity of the endotoxin was compared with *Escherichia coli* standard endotoxin (FDA, Bethesda, USA).

Inactivation and sterility control

Trivalent bulk products were tested for absence of live organisms by incubation of the product on gonococcal agar and Trypton Soy Broth (TSB) medium for 3 days at 37°C in a 5% CO_2 atmosphere.

To test sterility on final lot 5 ml was added to 180 ml Clausen and thioglycolate and incubated for a minimum of 13 days at 20–25°C for Clausen and 30–32°C for thioglycolate. No growth should be observed.

Immunological responses

Immunogenicity testing was performed in NIH mice. Eight week old female NIH mice were immunized subcutaneously with 10 μg total protein (1:10th human dose) of OMV vaccine with 2 mg ml^{-1} $AlPO_4$. After 28 days mice were euthanized and blood samples were collected.

Sera were tested for the presence of subtype specific antibodies using an OMV based ELISA, by immunoblotting and with a subtype specific bactericidal assay. Reference sera for all experiments were obtained from mice which were hyperimmunized with killed wild type bacteria.

For OMV based ELISA, plates were coated with a fixed concentration (0.025–0.1 μg total protein per well) of OMV derived from a single subtype [H44/76 (P1.7,16), 2996 (P1.5,2), MC51 (P1.19,15), 870227 (P1.5^c,10), 870446 (P1.12,13), 892257 (P1.7^h,4)] in PBS 0.01 M pH 7.2 overnight at room temperature. After washing serum samples were titrated in a ^{2}log dilution in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA and incubated at 37°C for 1 h. The binding of antibodies was demonstrated using biotinylated goat–anti-mouse-IgG (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 1 h and streptavidin–horseradish peroxidase (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 30 min. Binding of the conjugate was visualized using TMB/ethanol substrate.

Bactericidal antibodies were determined in subtype specific bactericidal assays. Bacterial strains as mentioned above were used to determine subtype specific antibodies except for P1.7^h,4 in which case 880049 was used as target for bactericidal antibodies. Briefly 25 μl of bacterial suspension (10^5 ml^{-1}) were incubated with baby rabbit complement (Pel-Freez Clinical Systems, Brown Deer, Wisconsin, USA) and ^{2}log dilutions of immune serum at 37°C for 1 h in a volume of 100 μl. Seven microlitres of this mixture was pipetted onto gonococcal agar plates in triplicate and incubated overnight at 37°C in a 5% CO_2 atmosphere. The serum bactericidal titre was reported as the highest reciprocal serum dilution yielding 50% killing of bacteria.

RESULTS

Production

Over a period of 2 years six independent OMV production runs were carried out, three productions

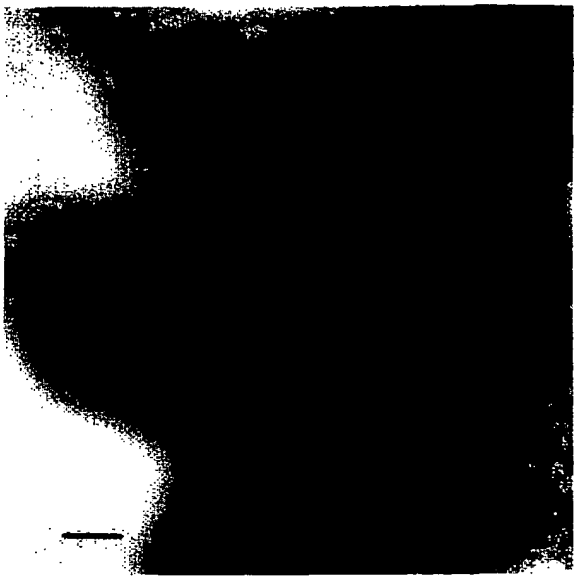

Figure 1 An electronmicrograph of a negative stained vesicle preparation of PL16215. The bar represents 100 nm

runs were performed of each experimental vaccine strain on a 135 l scale as described. After extraction and purification vesicles could be demonstrated in all preparations by negative contrast EM, an example of which is shown in *Figure 1*.

Total protein yield of the OMV production was determined after extraction and purification. Total protein yield ranged from *ca* 250 to 1000 mg in the final product. The differences in yield did not influence the composition or immunogenicity of the of the OMV (data not shown).

Vaccine characterization and identity tests

The composition of the OMV vaccine was characterized using different immunochemical and biochemical techniques.

As shown in *Figure 1* vesicles can be visualized using negative contrast staining and electron microscopy. OMV size ranged from 50 to 150 nm in different preparations. Intactness of the vesicles in these preparations ranged from 25 to 60% of the vesicles. The remaining vesicles were partly broken ("half moon structures"). Only very little amorphous granular material was found. Aggregates of vesicles were also observed frequently especially after the addition of sucrose.

Protein content was estimated by the method described by Peterson[21]. To demonstrate the presence of all relevant class 1 OMP epitopes an ELISA method was used. Vesicles or bacteria were coated on PVC plates and the presence of the relevant epitopes are characterized using subtype specific MoAbs (*Table 1*). The results of a typical assay are shown in *Figure 2*. All relevant epitopes can be demonstrated in both trivalent strains and in the OMVs which were made from these strains. In strain PL10124 OMV a reaction is observed of the $P1.7^h$ epitope with the P1.7 specific monoclonal, this reaction is normally absent in bacteria of the same strain (*Figure 2b*). It is known that this epitope is present in the original strain, but as a hidden epitope which cannot normally be detected[26]. It is clear from this figure that it is possible to discriminate between vesicles obtained from different strains. Immunogold electron microscopy using subtype specific monoclonal antibodies revealed that the relevant epitopes were present on the outside of the vesicles (*Figure 3*) and that vesicles derived from the two trivalent strains can be identified on basis of the class 1 protein (*Figure 4*).

When analysed on SDS–PAGE (*Figure 5*) the three class 1 OMPs which were expressed by PL16215 can be separated and their MW can be calculated. Using immunoblot analysis we have been able to demonstrate the origin of the three class 1 OMPs (data not shown). The MW of these proteins differ from that in the original strains due to the cloning procedure which is carried out[12]. Class 1 OMPs in PL10124 OMV can only be separated in two bands. The band with the lowest MW in PL10124 contains two different class 1 OMPs as was demonstrated by immunoblotting (data not shown). Class 4 and class 5 OMP are also present in the vesicles. A protein of 82 kD is also present in small amounts (<1%) in all OMV preparations.

The relative amount of class 1 OMP in the OMVs can be calculated using densitometry. Coomassie Brilliant Blue stained SDS-polyacrylamide gels were scanned using ImageMaster software. The relative amounts of the different OMPs in the OMV vaccine is given in *Table 2*. These results demonstrate that a relatively high portion of total protein in the vesicles is class 1 OMP which are responsible for the induction of bactericidal antibodies. The bactericidal antibodies follow the typical serosubtype characteristics. For clinical research two vaccine lots were prepared that contain 50 and 100 μg total protein per dose (0.5 ml). In *Table 2* the composition of the OMV vaccine of the 100 μg dose is given.

Taken together these results demonstrate that the vesicles are of the desired structure and composition i.e. contain intact proteins with the relevant epitopes in a high concentration.

Safety tests

Regarding the safety of the OMV the following parameters were considered.

- Inactivation of the bacteria which are used to prepare the OMV.
- Sterility of the intermediate and the end products.
- Determination of the toxicity related with the presence of bacterial endotoxin by LAL assay, LPS determination and pyrogenicity testing in rabbits.
- Abnormal toxicity of the hexavalent vaccine.

All safety tests were performed according to international guidelines or requirements [European Pharmacopeia (EP) and WHO]. For OMV based vaccines no specific requirements have been formulated by the EP or WHO. The only comparable vaccine is the Norwegian Folkehelsa vaccine based on H44/76 OMV[20]. This vaccine has been proven to be safe and effective.

Endotoxin determination

The bacterial LPS is a component of the bacterial outer membrane. Moreover, LPS is a structural component of the OMV and, although it is a toxic molecule, it

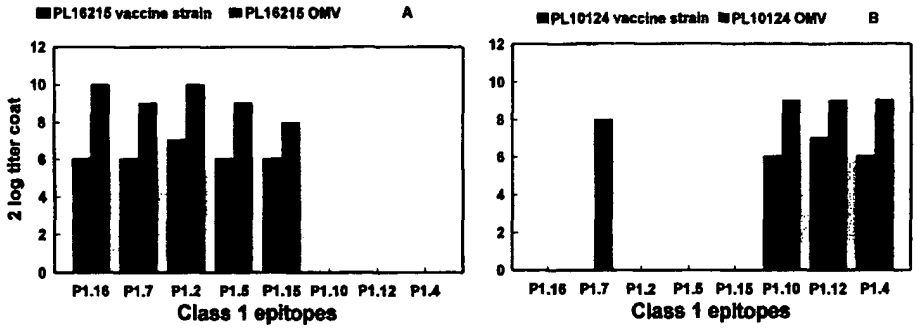

Figure 2 Presence of subtype specific epitopes in the two vaccine strains PL16215 and PL10124 and vesicles derived thereof as measured in ELISA. (A) Results obtained with PL16215 bacteria and OMV, (B) the results obtained with PL10124 bacteria and OMV

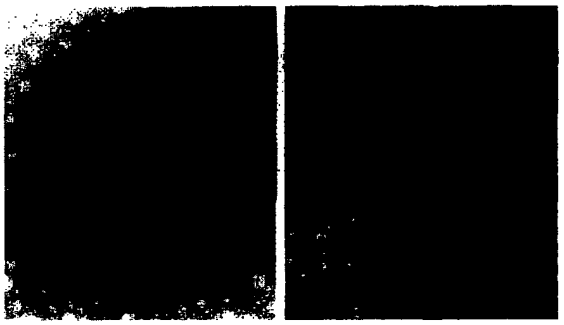

Figure 3 An electronmicrograph of an immunolabelled vesicle preparation of PL16215 (A). The P1.2 epitope is visualized on the surface of the vesicles by immunogold labelling using the monoclonal antibody MN16C13F4 (anti P1.2). Numerous 6 nm gold particles surround the vesicles (B). Control incubation with the monoclonal MN24H10.75 (anti P1.13) showed no gold particles. Bars indicate 100 nm

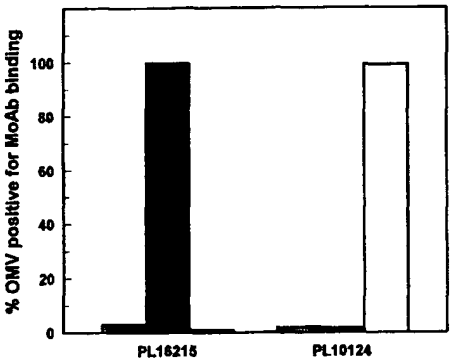

Figure 4 Vesicles of the PL16215 and PL10124 strains were labelled for P1.2 and P1.13 with the immunogold method. The percentage of OMV positive for the two class 1 epitopes is given. Approximately 300 vesicles were analysed per sample. ■, Control incubation without subtype specific MoAb; □, a-P1.2 incubation; and ■, a-P1.13 incubation. Almost 100% of the vesicles of each strain were positive for the correct class 1 epitope

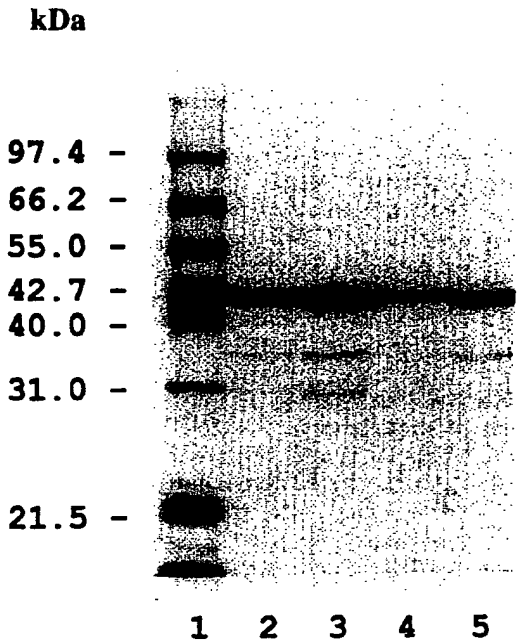

Figure 5 10% SDS–PAGE pattern of different vaccine bulk products. Class 1 OMP are *ca* 40 kDa. Lane 1, low molecular weight markers; lane 2, OMV PL16215 2 μg total protein; lane 3, OMV PL16215 4 μg total protein; lane 4, OMV PL10124 2 μg total protein; lane 5, OMV PL10124 4 μg total protein

Table 2 The composition of the hexavalent vaccine lot containing 100 μg of total protein: one human dose contains 0.5 ml

Substance	Concentration
Protein	200 μg ml^{-1}
Class 1 (6 subtypes)	89%
Class 4	7%
Class 5 (5c and 5.5)	3%
82 kDa	<1%
pH	6.9
DOC	22 μg ml^{-1}
B-PS	Absent
LPS (GalE)	20 μg ml^{-1}
$AlPO_4$	2 mg ml^{-1}
Sucrose	30 mg ml^{-1}
Thiomersal	0.7 mg ml^{-1}

is at the same time being studied as a possible vaccine adjuvant. Therefore we have carefully monitored the LPS content and the biological activity of the LPS in the vesicles. LPS free vesicles cannot be prepared since the presence of LPS in the OMV is necessary for the stability of the OMV vaccine. The LPS/protein ratio is lowered during the production process by partly replacing LPS by DOC. The LPS content was determined on the intermediate products by SDS–PAGE and silver-staining[24]. Results ranged from 2.5 to 10% LPS relative to the protein content. Both European Pharmacopoeia and WHO failed to provide requirements.

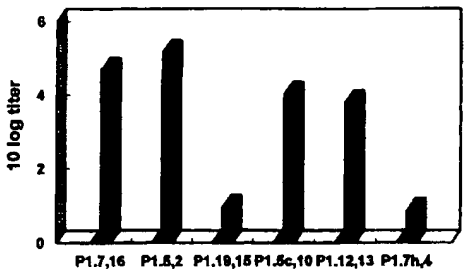

Figure 6 Immunogenicity of a hexavalent vaccine lot. The antibody response was measured in an OMV based ELISA against different subtype strains and is given as a reciprocal log titre of two fold serum dilutions. ▨, Pre-vaccination titres; ■, post-vaccination titres after 28 days

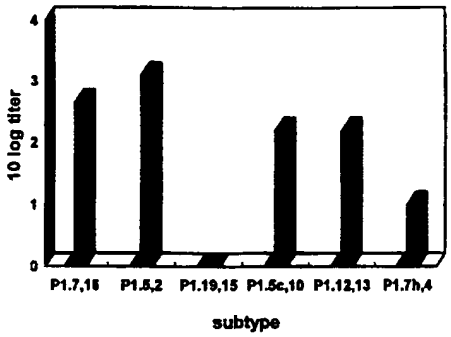

Figure 7 Bactericidal activity of sera of mice immunized with hexavalent vaccine lot. Results are given as reciprocal log titre of two fold serum dilutions. ▨, Pre-vaccination titers; ■, post-vaccination titres after 28 days

The biological activity of the endotoxin was determined in the limulus amoebocyte lysate (LAL) assay. Hexavalent final lot samples were tested in a fivefold dilution and contain 977 and 195 EU ml^{-1}, respectively, when compared with *E. coli* standard endotoxin. The endotoxin activity is within the range of DPT/polio vaccines and is therefore regarded as safe. Moreover, the endotoxin activity of LPS in vesicles is much lower than that of free purified LPS[20] (own observations).

To determine the pyrogenicity of the OMV vaccine a study in rabbits was performed. Samples of a hexavalent vaccine final lot (which contains the highest amount of LPS) were injected i.v. in rabbits (1 ml kg^{-1}) at 1:100, 1:300 and 1:1000 dilution and temperature rise was monitored. Furthermore a placebo which contains only aluminumphosphate and thiomersal, was injected at 1:100 dilution. For the 1:100 vaccine dilution a repeat of the test was necessary. The other dilutions passed the test. Temperature rises of 1:300 and 1:1000 dilutions were comparable with those obtained with the placebo (data not shown). 1:1000 dilution of the vaccine corresponds with the requirements for 23-valent pneumococcal polysaccharide vaccine and mono-, bi- and tetravalent meningococcal polysaccharide vaccines.

Immunological responses

Immunological responses were studied in NIH mice. Sera obtained from these mice at day 28 after immunization were analysed for the presence of anti meningococcal antibodies by ELISA, immunoblotting and bactericidal assays. Serial dilutions of sera from mice immunized with OMV vaccine revealed subtype specific responses in ELISA and bactericidal assays (*Figure 6* and *Figure 7*). Antibody responses against all individual subtypes which are included by the vaccine can be demonstrated on plates coated with vesicles derived from the original strains. Some epitopes seem to be more immunogenic than others. The P1.19,15 and $P1.7^{h}$,4 subtypes induced very low responses. Since an ELISA only demonstrates the elicitation of antigen binding antibodies but not of functional bactericidal antibodies the sera were also tested in a bactericidal assay. The results of these experiments are given in *Figure 7*. In this assay no anti-P1.19,15 (test strain) antibodies with bactericidal activity could be demonstrated, but all other strains were killed in the presence of complement.

In immunoblot experiments antibodies were demonstrated mainly directed at class 1 OMP but also against other OMP that were present in the vesicle (data not shown). The 82 kDa protein which was present only in small amounts (<1%) induced high antibody titres in mice as determined in immunoblotting. This reaction has also been observed in post-vaccination sera from humans after vaccination with H44/76 vesicles[27]

DISCUSSION

In the bacterial vaccine world, whole cells (inactivated or attenuated), toxoids and capsular polysaccharides (preferentially conjugated to proteins) are established vaccine principles. In addition to this there is a need for OMP based vaccines. Most research and clinical work is dedicated to meningococcal OMP vaccines. The OMV principle combines easy production procedures with potent delivery adjuvant characteristics. The OMP content of OMV vaccines can be manipulated by recombinant DNA technology and LPS in its various forms can be used as an adjuvant. Therefore, the meningococcal OMV vaccine deserves further attention as a meningococcal vaccine development and as a general principle.

We describe the preparation of an experimental hexavalent class 1 OMP containing OMV vaccine. Bacterial OMPs need to be presented in a membrane like structure to remain in or regain proper conformation. We have chosen an OMV formulation for vaccine purposes since this is a production friendly way of preparing immunogenic OMP containing vaccines. In our hands when OMV had disintegrated into AGM (amorphous granular material) the OMP were still immunogenic but the antibodies induced had very low bactericidal activity[28]. The likely explanation for this phenomenon is epitope competition at the B lymphocyte level, leading to a response against surface exposed epitopes (i.e. bactericidal antibodies) in case of an OMV vaccine and to a response against non-exposed epitopes in case of an AGM type vaccine. Alternative approaches for the OMV vaccine principles are artificial membrane like structures such as liposomes or ISCOMs (immune stimulating complexes). In that case the OMPs need to be purified and reintegrated into membrane like structures. Such procedures are more complicated in production terms as compared to the OMV principle.

We have used modified meningococci for our vaccine purposes. The vaccine strains did not express class 3 OMP, B polysaccharide and the lacto-*N*-neotetraose structure. In addition to class 1 OMP which represent *ca*

90% of the protein, class 4 and class 5 OMPs and some 82 kDa OMP are present in the vaccine. Each strain expressed three different class 1 OMPs. The advantages of combining three different class 1 OMP in one strain as compared to mixing OMV derived from three individual strains are obvious. The relative amount of class 1 protein is enhanced and thus it is possible to make a vaccine with a relatively low protein content (100 μg per dose) that contains a high amount of target protein. Moreover the LPS content in a vaccine that is a combination of six OMV preparations would be much higher than in the vaccine described.

The OMVs derived from these strains, were found to be stable over time (18 months) and after thermodegradation experiments (data not shown). In each strain class 1 OMPs are synthesized in equimolar amounts and OMVs of the two individual strains are mixed to contain equal amounts of all class 1 OMPs. The choice for the class 1 OMP was driven by findings in man and animals which demonstrate this protein to be a major target for bactericidal antibodies[29]. The heterogeneity of the class 1 OMP appears to be limited[10]. The P1.19,15 subtype in the trivalent OMV was non-immunogenic in the animal model tested for bactericidal antibody induction. Immunization of NIH mice with OMV containing only the P1.19,15 subtype and no other class 1 OMP did induce bactericidal antibodies (data not shown). The decreased immunogenicity of the P1.19,15 epitope in the trivalent vaccine in mice is now studied in our laboratory, however, an anti-P1.19,15 response could be demonstrated in some human adult volunteers i.e. fourfold rise in bactericidal antibody titre[18]. Whether it is immunogenic in children after a first immunization remains to be investigated.

The vaccines were also found to be safe for human use although LPS is present in a 10% w/w ratio as compared to OMP. It is known that the OMV structure has a shielding effect on the endotoxin properties of LPS[20]. Taken together the results with regard to safety of the vaccine do not deviate much from the data obtained with the Folkehelsa vaccine[20]. Since this vaccine proved to be safe in adolescents we do not expect adverse reactions on the basis of these observations.

A phase I study in adults with this vaccine has been carried out[18] and phase II studies in children and infants have started.

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